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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US91/04149 <b>(22) International Filing Date:</b> 12 June 1991 (12.06.91) <b>(71) Applicant:</b> GENPHARM INTERNATIONAL, INC. [US/US]; 2375 Garcia Avenue, Mountain View, CA 94043 (US). <b>(72) Inventors:</b> KRIMPENFORT, Paul ; Crayenestersingel 5, NL-2101 AN Heemstede (NL). LEE, Sang, He ; Sotaweg 24, NL-2371 GD Roelofarendsveen (NL). STRIJKER, Rein ; Spaargarenstraat 2, NL-2341 JW Oegstgeest (NL). <b>(74) Agent:</b> SMITH, William, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> EARLY DETECTION OF TRANSGENIC EMBRYOS  <b>(57) Abstract</b> <p>The present invention provides methods, based on in situ hybridization and the polymerase chain reaction, for the early detection of integrated transgenes in the nuclear genome.</p>		

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EARLY DETECTION OF TRANSGENIC EMBRYOS

5

## BACKGROUND OF THE INVENTION

In recent years there have been numerous reports of the production of transgenic animals, most of which relate to the production of transgenic mice. See, inter alia, U.S. Pat. No. 4,736,866; Andres et al., Proc. Natl. Acad. Sci. USA 84:1299-1303 (1987); Schoenberger et al., Experientia 43:644 (1987), and EMBO J. 7:169-175 (1988); and Muller et al., Cell 54:105-115 (1988), all incorporated herein by reference.

Several laboratories have also reported the production of transgenic porcine species (Miller et al., J. Endocrin. 120:481-488 (1989); Vize, P.D., et al., J. Cell Sci. 90:295-300 (1988); and Ebert et al., Mol. Endocrin. 2:277-283 (1988); all incorporated herein by reference), transgenic sheep (Nancarrow et al., Theriogenology 27:263 (1987); Clark et al., Bio/Technology 7:477-482 (1989) and Simons et al., Bio/Technology 6:179-183 (1988); all incorporated herein by reference), and rabbit (Hanover et al., Deutsche Tierarztliche Wochenschrift 94:476-478 (1987), incorporated herein by reference).

A number of reports have also suggested the production of transgenic cattle (e.g., Wagner et al., Theriogenology 21:29-44 (1984); Logse et al., Theriogenology 23:205 (1985); all incorporated herein by reference). A recent article has summarized the genetic engineering of livestock. (Pursel et al. Science 244:1281-1288 (1989); incorporated herein by reference). Methods for production of recombinant polypeptides in the milk of bovine species and methods for producing transgenic animals having desired phenotypes are discussed in co-pending U.S.S.N. 444,745 and 619,131; incorporated herein by reference.

High costs and long gestation periods of large animals make it difficult, time consuming and expensive to

produce transgenic livestock. Current techniques to produce transgenic animals involve transfer of all microinjected embryos to recipients and identification of transgenic animals among newborns. Only 1.3% and 10% of the newborns were transgenic in sheep and pigs, respectively (Hammer et al., Nature 315:680-683 (1985), incorporated herein by reference), and 5% of bovine fetuses were transgenic (Biery et al., Theriogenology 29:224 (1988), incorporated herein by reference).

Since the frequency of transgene incorporation in mammals is often low, and their gestation time long, the detection of transgene integration, e.g., in the preimplantation embryo would be highly desirable. Methods for early detection of transgene integration would significantly decrease the number of pregnancies required to produce a transgenic animal and substantially increase the likelihood that an implanted embryo will produce a transgenic animal. These methods would thus allow for far more efficient production of transgenic offspring, providing significant savings of time and resources. Such methods would be especially important for those animals for which very low or non-existent frequencies of transgenesis have been obtained, e.g. bovine species. The present invention fulfills these and other needs.

## SUMMARY OF THE INVENTION

The present invention provides methods for the early detection of integrated transgenes in the nuclear genome of animal embryos.

In one embodiment of the present invention, such integrated transgenes are detected by performing in situ hybridization on at least one metaphase stage cell from an embryo with a nucleic acid probe substantially complementary to the transgene. The presence of such an integrated transgene is detectable as a hybridization signal on both sister chromatids of a chromosome in the metaphase spread. Such a method is preferably nondestructive: a biopsy of at least one cell is removed from the embryo to be analyzed by in situ

hybridization, and the resulting biopsied cell is viable. The probe is typically labeled by biotinylation, and is preferably long, at least 2 kilobases in length to provide optimum sensitivity.

5 In another method of early detection of transgene detection in animal embryos, one takes advantage of the differences in DNA methylation patterns in procaryotes and eucaryotes. Integration and replication of a transgene methylated in a procaryotic fashion causes it to become  
10 methylated in a eucaryotic fashion. Such a change can be tested for by use of methylation sensitive enzymes. The restriction site of Dpn I, is methylated in Dam<sup>+</sup> strains of E. coli or may be methylated in vitro by dam methylase at N6 of an adenine of its restriction site. When integrated and  
15 replicated as part of the nuclear genome of an animal embryo, its restriction site loses its procaryotic methylation pattern, and can no longer be cleaved by Dpn I.

In one preferred method of the present invention, nuclear DNA from at least one cell from said embryo is treated  
20 with a restriction enzyme, such as Dpn I, capable of cleaving a methylated restriction site and incapable of cleaving said unmethylated restriction site, thereby producing restriction fragments of said nuclear DNA. These restriction fragments are amplified by PCR with PCR primers substantially complementary  
25 to sequences on different strands of the transgene and flanking the unmethylated restriction site. The amplified transgene may be detected only if the restriction enzyme has not cleaved its restriction sequence.

Alternatively, one may employ inverse PCR, cleaving  
30 the nuclear DNA of the embryo with a restriction enzyme incapable of cutting within the transgene, producing restriction fragments of said DNA; adding ligase to the restriction fragments under conditions which result in self ligation of the restriction fragments, thereby producing  
35 circularized restriction fragments; amplifying the circularized restriction fragments by inverse PCR, thereby producing an amplified transgene; and detecting the amplified transgene.

The embryos tested by these methods are typically bovine morulas at the 8 to 16 cell stage. It is preferable to clone the animal embryo prior to testing for an integrated transgene, such as by nuclear transfer.

5           Methods are also provided which additionally determine the sex of the embryo tested by the methods above described. Also embraced are embryos which have been produced by any of the methods of the present invention.

#### 10   DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for early detection of transgene integration in embryos which permit implantation and development to maturity of transgenic embryos so analyzed. Two basic approaches are provided: the first is  
15   based on the polymerase chain reaction (PCR), the second on the 'in situ' hybridization of nuclear DNA from embryonic cells with labeled probes. Each is described below in detail. These not only allow the early and rapid detection of transgene sequences in the cells of embryos into which transgene DNA has  
20   been introduced, but allow one to determine whether such DNA is truly integrated into the nuclear genome, giving rise to a transgenic animal, or is nonintegrated DNA which will eventually be lost. False positives, arising when nonintegrated DNA is mistakenly identified as integrated  
25   transgenes, would otherwise demand a significant expenditure of time and resources. The power of these techniques is enhanced when they are used in concert, preferably using PCR as an initial detection method, followed by in situ hybridization as a confirmatory method.

30

#### Cell and embryo culture; biopsies

Transgene sequences may be introduced into host cells in a variety of ways well known in the art, including: electroporation (Thomas and Capecchi, Cell 51:503-512 (1987) or  
35   microinjection of the transgene into the pronuclei of fertilized oocytes or nuclei of ES cells of the animal (Zimmer and Gruss, Nature 338:150-153 (1989); transfection of ES cells in culture by calcium phosphate precipitation (Gossler et al.,

Proc. Natl. Acad. Sci. USA 83:9065-9069 (1986); and infection of zygotes with a retrovirus containing the transgene (Jaenisch, Proc. Natl. Acad. Sci. USA, 73, 1260-1264 (1976); Robertson et al., Nature 323:445-448 (1986); all incorporated  
5 herein by reference).

Cells into which have been introduced transgene sequences are thereafter cultured in vitro until a "pre-implantation embryo" is obtained. Such pre-implantation embryos preferably contain approximately 16 to 150 cells. The  
10 8 to 32 cell stage of an embryo is commonly referred to as a morula. Those pre-implantation embryos containing more than 32 cells are commonly referred to as blastocysts. Blastocysts are generally characterized as demonstrating the development of a blastocoel cavity, typically at the 64 cell stage. Methods for  
15 culturing fertilized oocytes to the pre-implantation stage include, for example, those described by Gordon et al., Meth. Enzymol. 101:414 (1984); Hogan et al., in Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986) (for the mouse embryo); and Hammer et al.,  
20 Nature, 315:680 (1985) (for rabbit and porcine embryos); Gandolfi et al. J. Reprod. Fert. 81:23-28 (1987); Rexroad et al., J. Anim. Sci. 66:947-953 (1988) (for ovine embryos); and Eyestone et al., J. Reprod. Fert. 85:715-720 (1989); Camous et al., J. Reprod. Fert. 72:779-785 (1984); and Heyman et al,  
25 Theriogenology 27:5968 (1987) (for bovine embryos); all incorporated herein by reference.

Once the pre-implantation embryo stage is obtained, a biopsy is obtained, i.e., at least one cell is removed from the embryo. When equal division is used, the embryo is preferably  
30 not cultivated past the morula stage (32 cells). Division of the pre-implantation embryo (reviewed by Williams et al., Theriogenology 22:521-531(1986), incorporated herein by reference) may result in two "hemi-embryos" (hemi-morula or hemi-blastocyst), at least one of which is viable, i.e.,  
35 capable of subsequent development after implantation into the appropriate female to develop in utero to term. It is to be understood that such an embryo may be either equally or unequally divided into two hemi-embryos which are not

necessarily of equal cell number. Indeed, only one or two cells may constitute a biopsy from a single embryo; for that reason, one may more accurately speak of a "biopsied embryo" resulting from the removal of a biopsy of one or more cells from an embryo for analysis by the early detection methods provided herein. Essentially, all that is required is that one of the embryos which is not analyzed as hereinafter described be of sufficient cell number to develop to full term in utero.

The biopsied embryo, if shown to be transgenic, may be implanted in a female recipient animal to develop to term. In a preferred embodiment of the present invention, the methods for detecting transgenesis in pre-implantation embryos provided herein are combined with embryonic cloning steps to generate a population of transgenic embryos having the same genotype, several of which may provide cell biopsies for transgenesis detection. After transgenesis detection by the methods of the present invention, such cloned embryos may thereafter be implanted into recipient females to produce a population of transgenic animals also having the same genotype. Since only about 50% of biopsied embryos develop to birth upon transfer to a recipient, embryonic cloning ensures not only that enough biopsied cells are available to provide a conclusive test for transgenesis, but also that at least one of the cloned embryos shown to be transgenic will produce a transgenic animal.

Such embryo cloning may be performed by several different approaches. In one cloning method, the transgenic hemi-embryo is cultured in the same or in a similar media as used to culture individual oocytes to the pre-implantation stage. The "transgenic embryo" so formed (preferably a transgenic morula) is then divided into "transgenic hemi-embryos" which can then be implanted into a recipient female to form a clonal population of two transgenic animals. Alternatively, the two transgenic hemi-embryos obtained may be again cultivated to the pre-implantation stage, divided, and recultivated to the transgenic embryo stage. This procedure is repeated until the desired number of clonal transgenic embryos having the same genotype are obtained. Such transgenic embryos

may then be implanted into recipient females to produce a clonal population of transgenic animals.

In a preferred cloning method, the transgenic embryo is cloned by nuclear transfer according to the techniques of Prather et al. (Biol. Reprod. 37:59-86 (1988), incorporated herein by reference) and Roble et al. (J. Anim. Sci. 64:642-664 (1987); incorporated herein by reference). According to this method, nuclei from individual cells of the transgenic embryo are transplanted into enucleated oocytes, each of which is thereafter cultured to the blastocyst stage. At this point, the transgenic embryos may be subjected to another round of cloning or may be transferred to a recipient parent for production of transgenic offspring having the same genotype. By these means one may produce multiple, genetically identical embryos. One may obtain one or a small number of cells from each of these identical embryos to test for transgenesis by the methods of the present invention.

The term "transgenesis" is understood herein as meaning the integration of a transgene into the nuclear genome of a host organism, and its subsequent replication and stable maintenance as part of the nuclear genome. By "integration" is meant that the transgene is located on a chromosome, continuous with and linked by covalent bonds to the double stranded DNA which constitutes the chromosome. This situation may be contrasted with that of non-integrated extra-chromosomal DNA elements such as plasmids, episomes, and double minutes.

It is to be understood that transgenic embryos and/or transgenic animals having the same "genotype" means that the genomic DNA is substantially identical among the individuals of the embryo and/or transgenic animal population, having derived from the same genetic source. During mitosis, however, various somatic mutations may occur which may produce some variations in the genotype of one or more cells and/or animals. Thus, a population considered to have the same genotype may demonstrate individual or subpopulation variation.

Transgene detection using PCR

A variety of PCR methods are well known and widely practiced in the art. See, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and PCR Protocols: A Guide to Methods and Applications, Innis et al. eds., Academic Press, San Diego (1990), all incorporated herein by reference. Reagents and hardware for conducting PCR are commercially available through Perkin-Elmer/Cetus Instruments (PECI) of Norwalk, Connecticut.

Briefly stated, PCR is a cyclical process for the amplification of a nucleic acid template comprising the steps of: (a) denaturation of the template (generally by heating to 95°C to 100°C), (b) hybridization of dual oligodeoxynucleotide primers to the denatured template, and (c) template replication, consisting of an extension of these primers by a DNA polymerase (generally a thermostable polymerase such as Taq polymerase). Among the other reagents necessary for PCR are deoxynucleotide triphosphates (dNTPs, i.e., A, C, G, and T and their analogues) and a buffer to provide, for example, the salt and pH conditions necessary for optimal polymerase activity. The primers are so designed that they hybridize to sequences flanking the region to be amplified, one on each strand, and that the single strand generated by extension of each primer can act as a template for the extension of the other primer. Thus, each cycle of replication provides a two-fold amplification of the template. PCR can be thus be used to amplify target DNA sequences several million-fold. The amplified DNA can then be analyzed by restriction enzymes and electrophoresis.

In the methods of the present invention, oligonucleotides primers are synthesized that preferably hybridize specifically to sequences in the transgene, or, if present, to sequences in accompanying vector sequences. Upon amplification of the target sequence a DNA fragment of specific size will be generated.

A typical PCR buffer contains 10 to 50 mM Tris-HCl (between pH 8.3 and 8.8) when measured at 20°C, 50 mM KCl and 1.5 mM MgCl<sub>2</sub>. Modifications of this buffer may be necessary

for certain combinations of target nucleic acids and primers, as will be readily appreciated by those skilled in the art.

A number of suitable commercially available DNA polymerases are appropriate for performing PCR. Thermostable polymerases are preferred, such as those obtained from Thermus aquaticus (Taq polymerase) or T. flavis. For PCR or other amplification procedures, or for DNA sequencing reactions, other polymerases may be appropriate, including E. coli DNA polymerase I or its Klenow fragment, reverse transcriptase, phage T4 or T7 polymerases, or structural variants and modified forms of these and other polymerases.

The sensitivity of PCR is particularly useful for analyzing small DNA samples (e.g. from a small number of cells). This sensitivity can, however, be problematic unless stringent precautions are taken to avoid specific contamination. DNA contamination from aerosol particles, pipettes, primers, etc. can be a big problem when the DNA used for injection of the embryos is prepared in the same lab where PCR is performed. Furthermore, PCR cannot discriminate between integrated DNA and non-integrated DNA. The following stringent measures can prevent contamination: PCR analyses in a completely isolated lab equipped with a flow hood with filter; special equipment (pipettes, microfuges, etc.) dedicated to PCR; all reagents (primers, buffers, Taq polymerase) supplied in kits that can be opened and used only once.

The problem of discriminating integrated from non-integrated DNA constructs may be solved by taking advantage of the difference in methylation patterns between DNA replicated in procaryotes and DNA replicated in eucaryotes. DNA constructs which have been cloned in procaryotes and/or methylated in vitro will retain the procaryotic methylation profile, unless they integrate in and, consequently, are replicated as part of the host genome. For example, the A of the sequence GATC, the recognition site for the restriction enzyme Dpn I, is methylated in Dam<sup>+</sup> strains of E. coli; it is unmethylated in eukaryotic cells, which lack the dam methylase. Eukaryotic DNA, on the other hand, is methylated at G in the sequence GC. After several cleavages, the integrated

transgene, having been replicated along with the nuclear genome, will be methylated in a eucaryotic fashion. By digestion prior to PCR with restriction endonucleases capable of cutting methylated restriction sites but not nonmethylated restriction sites (e.g., Dpn I), it should be possible to  
5 eliminate nonintegrated DNA while leaving the integrated, i.e. restriction endonuclease resistant, DNA available for amplification. Thus, only embryos having an integrated transgene should give rise to an amplified band. It should  
10 also be noted that the use of such a restriction enzyme also reduces the problem of contamination by bacterial DNA (e.g., plasmid) contamination.

It will be apparent to those skilled in the art that one may likewise employ other methylation sensitive enzymes  
15 which can cut sequences in the transgene which display the eukaryotic methylation pattern but which fail to cut the same sequences when they display a prokaryotic methylation pattern. For example, one may first amplify the transgene by PCR, then digest the amplified DNA with such a methylation sensitive  
20 enzyme having one or more restriction sites within the transgene (i.e., between the sites to which the two PCR primers hybridize). One would then detect on a stained gel or Southern blot of the PCR amplification products two (or more) DNA fragments resulting from the digestion of the single amplified  
25 DNA product.

An alternative method for detecting integrated transgenes, "inverse PCR", makes use of primers which have opposite (reverse) orientation (Triglia et al., Nucl. Acids Res. 16:8186 (1988); Ochman et al., Genetics 120:621-623  
30 (1988), both incorporated herein by reference). The DNA isolated from biopsies is cut with restriction enzymes and the fragments are circularized by ligation. In biopsies from non-transgenic embryos, DNA fragments of expected length are amplified (derived from concatamers), while DNA from transgenic  
35 embryos will also give rise to fragments of varying size. These fragments contain host sequences which are present between the reverse primers in the religated circles.

Since the DNA introduced into mammals ("transgene") may be quite long (up to 50 kb or more, and typically 20 kb or more) and often lack vector sequences, PCR primers are preferably designed to be complementary to sequences within the transgene itself.

A preferred method for detecting transgenesis by PCR at an early stage in the embryo's development employs a methylation sensitive restriction endonuclease such as Dpn I. Dpn I recognizes and cleaves the sequence GATC in double stranded DNA only when the adenine in each strand within this sequence is methylated at N-6. In this preferred method, the transgene containing the sequence GATC is methylated prior to microinjection either by transferring the transgene on an appropriate plasmid to a Dam<sup>+</sup> strain of a microorganism such as E. coli MM294, or by directly methylating the transgene in vitro with dam methylase (commercially available from a number of vendors, including New England Biolabs). The methylated transgene (preferably without any exogenous sequences such as plasmid vector) is then microinjected into fertilized oocytes (approximately 10 to 500 copies per pronucleus, more preferably 50 to 100 copies per pronucleus). The fertilized oocytes so obtained are cultured in vitro to the pre-implantation stage. During this early growth and cell division phase, the genomic DNA is replicated. Accordingly, those copies of the methylated transgene integrated into the genome of the fertilized oocyte are unmethylated after replication, whereas any non-integrated transgenes which may still exist after replication will remain methylated (Lacks et al., J. Mol. Biol. 114:153 (1977), incorporated herein by reference). This differential methylation pattern for an integrated versus a non-integrated transgene permits the identification of which fertilized oocytes have integrated the transgene into the genome.

The identification of the pre-implantation embryos containing the integrated transgene is achieved by analyzing the DNA from each of the embryo biopsies (or the pooled biopsies from several genetically identical embryos arising from cloning a single embryo). Such DNA is typically obtained by lysing the biopsied cells and analyzing the released DNA

after treatment as described by Ninomiya et al., Molecular  
Reproduction and Development 1:242-248 (1989), incorporated  
herein by reference. Each of the DNA samples is treated with  
Dpn I. Thereafter, PCR is used to amplify all or part of the  
5 transgene. When the entire transgene is amplified, two  
extension primers each complementary to opposite strands at  
opposing ends of the transgene are used. When, however, less  
than the entire transgene is amplified, such extension primers  
are chosen such that the amplified gene product spans the Dpn I  
10 site in the transgene. That is, the primer binding sites for  
the two PCR primers flank the Dpn I site. If Dpn I cleavage  
has not occurred, PCR amplification will result in amplified  
sequences having a predicted size, whereas no amplification  
product will result for those transgenes which have been  
15 cleaved. Generally, the Dpn I digested and PCR amplified DNA  
from the biopsy is subjected to electrophoresis. Although it  
is often possible to simply visualize the amplified DNA on an  
ethidium bromide stained gel, electrophoresis is preferably  
followed by hybridization with labeled probe complementary to  
20 the region of the transgene between the two extension primers.  
By these means the size of the amplified DNA sequences, if any,  
is determined. The presence of an amplified sequence of the  
appropriate size indicates whether the transgene has been  
integrated into the pre-implantation embryo from which the  
25 biopsy was obtained (now called a "transgenic biopsied  
embryo").

If integration is detected, the remaining untreated  
transgenic biopsied embryo is transplanted into a recipient  
parent. After in utero development the transgenic animal  
30 having the desired phenotype conferred by the integrated  
transgene is identified by an appropriate method in utero or  
after birth.

The use of Dpn I, of course, requires that the  
sequence GATC be present in the transgene of interest. In  
35 those cases when such a sequence is not present, it may be  
readily introduced into the transgene by site directed  
mutagenesis (Kunkel, Proc. Natl. Acad. Sci. USA 82:488 (1985),  
incorporated herein by reference) or cassette mutagenesis

(Wells et al., Gene 34:315 (1985), incorporated herein by reference), provided such mutagenesis does not change the amino acid sequence encoded by the transgene (or causes an inconsequential change in amino acid sequence) and that any  
5 codons so generated are functional in the transgenic animal of interest.

In an alternate embodiment, the above described method for detecting transgenesis in pre-implantation embryos is combined with embryonic cloning steps to generate a  
10 population of transgenic embryos having the same genome type which may thereafter be implanted into recipient females to produce a population of transgenic animals also having the same genotype. In this regard it is to be understood that transgenic embryos and/or transgenic animals having the same  
15 "genotype" means that the genomic DNA is substantially identical between the individuals of the embryo and/or transgenic animal population. It is to be understood, however, that during mitosis various somatic mutations may occur which may produce some variations in the genotype of one or more  
20 cells and/or animals. Thus, a population having the same genotype may demonstrate individual or subpopulation variations.

To detect the PCR amplified products, e.g., a sample is electrophoresed on an agarose or polyacrylamide gel.  
25 Smaller DNA fragments resulting from amplification are preferably analyzed by polyacrylamide gel electrophoresis. DNA bands are visualized by ethidium bromide staining, or, to increase sensitivity, gel electrophoresis can be followed by Southern blotting or dot blotting and hybridization with a  
30 labeled probe by techniques well known in the art. Also, labeled PCR primers, which are incorporated into the amplified DNA product, may be employed by methods well known in the art to assist in visualization of the amplification product and thus increase sensitivity.

35

#### Transgene detection by in situ hybridization

Techniques have been developed in which nucleic acid probes are used to locate specific complementary nucleic acid

sequences, e.g., specific DNA sequences on intact chromosomes, in situ, a procedure called "in situ hybridization". Labeled nucleic acid probes of a predetermined nucleotide sequence are hybridized to the chromosomes of sample cells (or tissues), the DNA double strands of which have been denatured by a brief exposure to a very high pH. The chromosomal regions that bind the probe during the hybridization step are examined by microscopy to determine whether they hybridize to the probes and thus contain the specific nucleic acids of interest.

Beside the use of radionuclide labeled nucleic acid probes, there are two distinct non-radioactive hybridization methods: the direct and the indirect methods. In the direct methods, the reporter molecule is bound to the nucleic acid probe so that the molecular hybrids between probe and target sequences can be visualized microscopically immediately after the in situ hybridization procedure. Such methods include the terminal fluorochrome labeling procedure of RNA probes (Baumann et al., 1980, 1984) and the direct enzyme labeling procedure of nucleic acids (Renz and Kurz (1984).

For indirect procedures, the probe must contain an element, introduced chemically or enzymatically, that renders it detectable by affinity cytochemistry, hence the term indirect. Again, the presence of such an element should not, or only in a limited way, interfere with the actual hybridization reaction and the stability of the resulting hybrid. A number of such hapten modifications, including acetylaminofluorene, mercury, biotin and transamination procedures, have been described (Langer et al., Proc. Natl. Acad. Sci. USA 78:6633-6637 (1981); Leary et al., Proc. Natl. Acad. Sci. USA 80:4045-4049 (1983); Landegent et al., Exp. Cell Res. 153:61-72 (1984); Tchen et al., Proc. Natl. Acad. Sci. USA 81:3466-3470 (1984); Hopman et al., Histochemistry 84:169-178 (1986); Hopman et al., Histochemistry 84:179-185 (1986); Hopman et al., Nucl. Acids Res. 14:6471-6488 (1986); Shroyer and Nakane, J. Cell Biol. 97:377a (1983); Van Prooijen-Knegt et al., Exp. Cell Res. 141:397-407 (1982); Raap et al., Histochemistry 81:517-520 (1984); Viscidi et al., J. Clin. Microbiol. 23:311-317 (1986); Rudkin and Stollar, Nature

265:472-473 (1977); see for review Raap et al., In Techniques in Immunochemistry, vol. IV, Bullock and Petrusz, eds., New York, Academic Press, pp. 167-197 (1989); all incorporated herein by reference). When antibodies against the reporter  
5 molecules are available, then direct methods are also amenable to immunocytochemical amplification (Bauman et al., Histochemistry 73:181-193 (1981); Lansdorp et al., J. Histochem. Cytochem. 32:172-178 (1984); Pinkel et al., Proc. Natl. Acad. Sci. USA 85:2934-2938 (1986); all incorporated  
10 herein by reference).

Recently, the chemical synthesis of oligonucleotides containing functional groups (i.e. primary aliphatic amines or sulfhydryl groups) has been described, thus allowing in principle the application of nucleotide oligomers in non-  
15 radioactive in situ hybridization by coupling haptens (e.g., biotin or digoxigenin) or reporters like fluorochromes or enzymes (Agrawal et al., Nucl. Acids Res. 14:6227-6245 (1986); Chollet and Kawashima, Nucl. Acids Res. 13:1529-1541 (1985); Haralambidis et al., Nucl. Acids Res. 12:4857-4876 (1987);  
20 Jablonski et al., Nucl. Acids Res. 14:6115-6129 (1986); Kempe et al., Nucl. Acids Res. 13:45-57 (1985); Ruth, DNA 3:123-129 (1984); all incorporated herein by reference).

Labeling DNA probes chemically instead of radioactively improves the spatial resolution of this  
25 technique. Preferably, the probes are synthesized with nucleotides that contain a biotin side chain, and the hybridized probes are detected by staining with a network of streptavidin and some type of marker molecule. With this technique it is possible to detect specific, single copy  
30 nucleic acid sequences in individual cells and on individual chromosomes (See for review: Raap et al., In Techniques in Immunocytochemistry, vol. IV, ed. G. Bullock and P. Petrusz: New York, Academic Press (1990), incorporated herein by reference).

35 The advantage of the use of metaphase chromosomes for in situ hybridization is that it enables the discrimination between integrated and non-integrated DNA. Nonintegrated DNA molecules will probably be present in high numbers, fused

together in one or a few large concatamers, yet diffused randomly throughout the nucleus and not associated directly with a chromosome. Integrated DNA will be visualized as discrete spots at the same location on two sister chromatids.

5 Initially, in situ hybridization methods used DNA or RNA probes labeled directly with a terminal fluorochrome. The sensitivity of these direct methods was low. Therefore, immunocytochemical amplification procedures have been developed to increase detection sensitivity. Several chemical and  
10 enzymatic nucleic acid hapten modifications are now available for the amplification on DNA or RNA probes for in situ hybridization (Raap et al., 1989). All modifications have in common that the hapten does not affect the hybridization properties of the probes. Most haptenized probes are detected,  
15 after hybridization, with antibodies specific for the hapten.

Although in situ hybridization is a fairly complex multistep procedure in which each step must be optimized, the technique has been successfully employed, for example, in the diagnosis of viral infection (Brigati et al., Virology 126:32-  
20 50 (1982); Burns et al., J. Clin. Pathol. 39:1066-1073 (1986); Raap et al., Histochemistry 88:367-373 (1988); all incorporated herein by reference) and the analysis of gene expression at the mRNA level (Rudkin and Stollar, Nature 265:472-473 (1977), incorporated herein by reference); for the assessment of  
25 chromosome copy number in early prenatal diagnosis (Julien et al., Lancet ii:863-864 (1986), incorporated herein by reference) and karyotyping of tumor cells (Devilee et al., Cancer Res. 48:5825-5830 (1988); Hopman et al., Histochemistry 89:307-316 (1988); all incorporated herein by reference).  
30 Heretofore, however, it has not been employed for the detection of incorporated transgenes in early embryos.

Currently, the sensitivity of the haptenized probes is sufficient for single copy gene localization on metaphase nuclei (Albertsen, EMBO J. 4:2493-2498 (1985); Bhatt et al.,  
35 Nucleic Acids Res. 42:42:153-161 (1988); Landegent et al., Nature 317:175-177 (1985); Lawrence et al., Cell 52:51-61 (1988); all incorporated herein by reference).

Further testing to confirm transgenesis and sex determination

In a preferred embodiment of the present invention, PCR will be employed to provide an initial determination whether the transgene is incorporated into the genome of an embryo, while in situ hybridization is used to confirm the results of the initial testing. Alternatively, PCR may be used to confirm the results obtained by an initial test using in situ hybridization. The sex of the embryos so tested may further be sexed by PCR or in situ hybridization using probes specific for the Y chromosome, or by other methods well known in the art.

To follow up and provide further confirmation for the methods of the present invention for detecting early transgenesis, other methods may be used, including in utero and post partum analysis of tissue. In utero analysis is performed by several techniques. In one such technique, transvaginal puncture of the amniotic cavity is performed under echoscopic guidance (Bowgso et al. (1975) Bet. Res. 96:124-127; Rumsey et al. (1974) J. Anim. Sci. 39:386-391; both incorporated herein by reference). This involves recovering about 15 to 20 milliliters of amniotic fluid between about day 35 and day 100 of gestation. This volume of amniotic fluid contains about 1000 to 12,000 cells per ml originating from the urogenital tract, the skin, and possibly the lungs of the developing embryo. Most of these cells are dead. Such cells, however, contain genomic DNA which is subjected to PCR analysis for the transgene as an indication of a successful transgenesis. Alternatively, fetal cells may be recovered by chorion puncture. This method also may be performed transvaginally and under echoscopic guidance. In this method, a needle is used to puncture the recipient animal's placenta, particularly the placentonal structures, which are fixed against the vaginal wall. Such sampling may be performed around day 60 of gestation. Chorion cells, if necessary, are separated from maternal tissue and subjected to PCR analysis for the transgene as an indication of successful transgenesis.

Transgenesis may also be detected after birth. In such cases, transgene integration can be detected by taking an

appropriate tissue biopsy such as from the ear or tail of the putative transgenic animal. About one to two centimeters of tail or about five to ten square millimeters of ear are obtained, followed by Southern blotting with a probe for the transgene according to the method of Hogan et al. (1986) 5 Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory, incorporated herein by reference.

#### Recombinant DNA techniques

10 It will be readily apparent that the present invention relies heavily upon a thorough knowledge and understanding of recombinant DNA technology. The recombinant DNA techniques employed when using the present invention are well established and constitute recognized methods, e.g., for 15 the use of restriction endonucleases; for the preparation of predetermined nucleotides in sequence as hybridization probes; and for the various methods of labelling such DNA or RNA probes using a variety of labels, such as radionuclides. Accordingly, it is presumed that one practicing the present invention is 20 familiar with the applications and limits of the various techniques and will recognize that minor changes in reagents, concentrations, temperature, reaction times and similar alterations of known methods are merely obvious variations of choice. Any major differences from the published and well 25 accepted techniques will be identified and described in detail as necessary. However, no description or repetition of the many compositions, protocols and manipulative techniques will be given here for these well known procedures. A complete description and recitation of the theory and practice of 30 various recombinant DNA techniques, as well as various reagents, experimental parameters and other practical considerations, one may consult, inter alia, Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or Current Protocols in 35 Molecular Biology, F. Ausubel et al., ed. Greene Publishing and Wiley-Interscience, New York (1987), which are incorporated herein by reference.

In addition it is presumed and understood that the terminology, technical and otherwise, used herein follows their usual, well understood meanings as they are used and applied in common parlance or the technical literature. For these reasons, only if a term has not been used previously within the field generally or if an unusual definition is employed, then and then only will a formal definition be provided as part of the text. For this reason, such technical terms as nucleotide, nucleic acid, genome, vector, and similar technical terms are presumed to be commonly understood by the practitioner ordinarily skilled in this art.

As used herein, the term "transgene" refers to a DNA sequence which is capable of producing a desirable phenotype when contained in the genome of cells of a transgenic animal. Such a transgene often comprises a recombinant DNA sequence encoding a "recombinant polypeptide". In such cases, the transgene is capable of being expressed to produce the recombinant polypeptide. Unless otherwise noted, the term also is used to embrace vector sequences and non-coding flanking sequences which may accompany such polypeptide-encoding sequences.

#### PCR Primers and Nucleic Acid Probes

The PCR primers used in the methods of the present invention are oligonucleotides, whether occurring naturally as in a purified restriction digest, or produced synthetically, which are capable of hybridizing specifically to a known sequence in a target gene. When a PCR primer, especially the terminal 3' nucleotide of the primer, has hybridized, it acts as a point of initiation of synthesis under conditions in which synthesis of a primer extension product is favored. Such conditions typically include the presence of four different nucleotide triphosphates and a thermostable polymerase in an appropriate buffer and at a suitable temperature.

Such primers are preferably single stranded for maximum efficiency and amplification, although double stranded primers may be employed if treated to separate the complementary strands before use. The oligonucleotides

employed as primers may contain naturally occurring nucleotides or their analogs, such as 7-deazaguanosine or inosine, and may be either DNA or RNA.

Such an oligonucleotide will preferably contain at least one or preferably more than one region of eight or more consecutive nucleotides having perfect complementarity with the target sequence. They will also usually have one or more nucleotides at the 3' end displaying perfect complementarity (i.e., base pairing) with a single stranded region of a target nucleic acid. However, longer oligonucleotides (e.g., more than 20 nucleotides) may anneal with the desired specificity, even though no such region of consecutive nucleotides with perfect complementarity may be present.

The primer sequence need not, however, reflect the exact sequence of the template, and may include sequences in addition to those allowing the primer to hybridize with specificity to the template. Alternatively, noncomplementary bases or longer sequences can be interspersed into the primer provided that the primer sequence has sufficient complementarity with the sequence of the target sequence to hybridize with it and thereby allow synthesis of the extension product. In addition, the primer may include nucleotides which have been substituted, e.g., with biotin. As a consequence of amplification by PCR, the sequence and substituents of the primer are introduced into the amplified product.

Once the appropriate primer sequences are determined, they are preferably synthesized using commercially available methods and equipment. Synthetic oligonucleotides can be produced by the solid phase phosphoramidite method according to Caruthers et al., Cold Spring Harbor Symp. Quant. Biol., 47:411-418 (1982), and Adams et al., J. Am. Chem. Soc., 105:661 (1983), or the triester method according to Matteucci, et al., J. Am. Chem. Soc., 103:3185 (1981), all incorporated herein by reference.

Preferably the oligonucleotide is an oligodeoxyribonucleotide. Its exact length will depend on such factors as number of mismatches, if any, temperature, salt conditions, and other parameters discussed above.

Oligonucleotide primers typically are from about 8 to 50, usually about 12 to 50, and preferably 16 to 30 nucleotides in length, although longer or shorter primers may be appropriate.

Suitable probes, e.g., for in situ analysis or for  
5 analyzing Southern blots of the products of PCR amplification, may be RNA or DNA and may be either double stranded or single stranded. In situ hybridization probes will preferably be as long as possible (greater than 2 kb is preferable) to increase sensitivity, although shorter probes may be used, and will  
10 preferably be complementary to exon sequences in a transgene to reduce the chance for nonselective hybridization. Probes for Southern blots may be shorter probes substantially complementary to sequences in a transgene. The nucleic acid probes may be derived from genomic DNA or cDNA, prepared by  
15 chemical or enzymatic synthesis (e.g., as an RNA transcribed from a template sequence comprising a sequence substantially complementary to a transgene), or may be a hybrid of the various combinations. Recombinant nucleic acids comprising sequences otherwise not naturally occurring may also be  
20 employed. They may contain naturally occurring nucleotides or their analogs, such as 7-deazaguanosine or inosine.

Such probes may be labeled by any of the methods commonly used in the art, such as nick translation or random hexamer labeling. Such nucleic acid probes will include an  
25 isolated nucleic acid attached to a label or reporter molecule. Probes may be prepared by nick translation, Klenow fill-in reaction, random hexamer priming, or other methods known in the art. For isolating nucleic acids, choosing label or reporter molecules, labeling probes, and other aspects of probe  
30 preparation see, inter alia, Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989), Current Protocols in Molecular Biology, F. Ausubel et al., ed. Greene publishing and Wiley-Interscience, New York (1987), incorporated herein by  
35 reference.

Substantial homology or complementarity; Selective and stringent hybridization

An oligonucleotide primer or a probe is functionally defined as "substantially homologous" or "substantially complementary" to a target nucleic acid when it will anneal or hybridize to a single desired position on a strand of the nucleic acid being targeted or its complementary strand such that stable and specific binding occurs between the primer and the target nucleic acid under selective conditions (See, M. Kanehisa, Nucleic Acids Res. 12:203 (1984), incorporated herein by reference). Selectivity of hybridization exists when hybridization occurs which is more selective than total lack of specificity, and is generally marked, in the methods of the present invention, by the formation of a single desired extension product.

Proper annealing conditions depend, for example, upon an oligonucleotide's length, base composition, and the number of mismatches and their position on an oligonucleotide, or on the annealing temperature, salt concentration of the medium, and other conditions, and must often be determined empirically. For discussions of oligonucleotide design and annealing conditions, see, for example in Sambrook et al. (1989) or F. Ausubel et al., ed. (1987), which are incorporated herein by reference.

Stringent primer annealing conditions will vary with the specific application, but typically include salt concentrations of less than about 1 M, more usually less than about 500 mM and preferably less than about 200 mM. Temperature conditions will typically be greater than 22°C, more typically greater than about 30°C and preferably in excess of about 37°C. As other factors may dramatically affect the stringency of hybridization, including base composition and size of the complementary strands, the presence of such salts as  $MgCl_2$ , and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one alone.

Alternatively, on the basis of sequence comparison, an oligonucleotide primer or a probe is considered

"substantially homologous" or substantially complementary") to a target sequence whenever the primer and target sequence, or their complementary strands, when optimally aligned and compared, are identical with appropriate nucleotide insertions or deletions, generally in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about 98 to 99.5% of the nucleotides.

The invention will better be understood by reference to the following examples, which are intended to merely illustrate the best mode now known for practicing the invention, but the invention is not to be considered limited thereto.

### EXAMPLES

15

#### 1. In vitro Maturation, Fertilization and Culture of Bovine Oocytes

Immature oocytes are obtained in large quantity (400-600/day) by aspirating follicles of ovaries obtained at abattoirs. Immature oocytes are cultured for a period in vitro before they are competent to be fertilized. Once "matured", oocytes are fertilized with sperm which has also been matured, or "capacitated" in vitro. The pronuclei of the fertilized oocyte is then injected with the transgene encoding for the expression and secretion of human lactoferrin. Zygotes resulting from this in vitro fertilization and microinjection are then cultured to the late morula or blastocyst stage in media with somatic tissue, or in medium "conditioned" by somatic tissue. Blastocysts are then transferred non-surgically to recipient cattle for the balance of gestation or analyzed for integration of the transgene as described herein.

In vitro maturation (IVM). Ovaries are obtained immediately after slaughter at local abattoirs and oocytes are recovered. Alternatively, oocytes are obtained from living cattle by surgical, endoscopic, or transvaginal ultrasonic approaches. In all cases, oocytes are aspirated from ovarian follicles (2-10 mm diameter). After washing, oocytes are placed in a maturation medium capable of supporting nuclear and

cytoplasmic maturation of bovine oocytes. Examples of such media are given by Sirard et al. (Biol. Reprod. 39:546-552 (1988), incorporated herein by reference).

In vitro fertilization (IVF). Matured oocytes are  
5 fertilized with either fresh or frozen thawed sperm. Sperm are then added to a fertilization media consisting of a modified Tyrode's solution (Parrish et al. (1986) supra., incorporated herein by reference) supplemented with heparin to induce sperm capacitation (Parrish et al., Biol. Reprod. 38:1171-1180  
10 (1988), incorporated herein by reference). Capacitation constitutes the final sperm maturation process which is essential for fertilization. Sperm and oocytes are co-cultured for 18 hours. A useful feature of this IVF method is that (in the case of frozen sperm) consistent, repeatable results are  
15 obtained once optimal fertilization conditions for a particular ejaculate have been defined (Parrish et al. (1986) supra., incorporated herein by reference).

In vitro culture (IVC). Conventional culture systems, which support development of mouse, rabbit, or human  
20 ova, do not support development of bovine embryos past the 8-16 cell stage. This problem has been overcome by pre-conditioning culture media with oviductal tissue. Oviduct-conditioned medium will support bovine embryos past the 8-16 cell stage to the blastocyst stage in vitro (Eyestone and First, J. Reprod.  
25 Fert. 85:715-720 (1989), incorporated herein by reference).

Bovine embryos have proven refractory to in vitro culture. This in part stems from the existence of a "block" to cleavage in vitro at the 8-16 cell stage. This block may be alleviated by culturing embryos in the oviducts of rabbits  
30 (reviewed by Boland, Theriogenology 21:126-137 (1982), incorporated herein by reference) or sheep (Willadeen in: Mammalian Egg Transfer (E. Adams, ed.), pp. 185-210 (1982); Eyestone et al., Theriogenology 28:1-7 (1987); both incorporated herein by reference). However, these in vivo  
35 alternatives have been less than ideal, in that: (1) they require the maintenance of large numbers of recipient animals, (2) they require surgery to gain access to the oviducts for transfer, and a second surgery (or sacrifice) to recover the

embryos, (3) all transferred embryos are seldom recovered, and (4) access to embryos during culture for observation or treatment is entirely precluded. The lack of in vitro culture systems has hampered the development of various manipulation techniques (such as gene transfer by pronuclear injection) by preventing accumulation of basic information of the chronology and ontogeny of bovine development, and by complicating the process of culturing embryos to a stage compatible with non-surgical embryo transfer and cryopreservation techniques (e.g., late blastocyst stages).

Bovine embryos did not yield to attempts to culture them in vitro past the 8-16 cell "block" until Camous et al., J. Reprod. Fert. 72:479-485 (1984), incorporated herein by reference) demonstrated cleavage to 216 cells when embryos were co-cultured with trophoblastic tissue.

The co-culture procedure was extended to oviductal tissue, based on the ability of homo- or hetero-oviducts to support development from zygote to blastocyst. Thus, bovine embryos co-cultured with oviductal tissue, or in medium conditioned by oviductal tissue, developed from zygote to blastocyst in vitro (Eyestone and First, J. Reprod. Fert. 85:715-720 (1989); Eyestone, "Factors affecting the development of early bovine embryos in vivo and in vitro." Ph.D. Thesis, University of Wisconsin (1989), both incorporated herein by reference). Blastocysts have been produced in this system after superovulation and artificial insemination, or by in vitro maturation (IVM), and fertilization (IVF) of immature oocytes. Blastocysts produced in this fashion resulted in pregnancies and live calves after transfer to recipient animals. The results obtained were as follows:

<u>Step</u>	<u>Efficiency</u> <u>(%)</u>	<u>Number</u> <u>(per 100)</u>
35	IVM	90
	IVF	72
	IVC	22
40	Embryo transfer (% pregnant)	11

Therefore, from an initial daily harvest of 500 oocytes, it is expected the approximately 55 pregnancies will result.

Preparation of Oviduct Tissue and Use For Co-Culture and Conditioned Medium. Ovine oviducts are obtained after slaughter or by salpingectomy. The luminal tissue is harvested by scraping an intact oviduct gently with a glass slide and washed five times in 10 ml modified tyrodes-hepes solution (Parrish et al., Biol. Reprod. 38:1171-1180 (1988), incorporated herein by reference). The final tissue pellet is suspended in M199 + 10% fetal calf serum at a ratio of 1 volume tissue to 50 volumes of media.

The tissue suspension can be used for embryo-co-culture. Alternatively, media may be conditioned for 48 h, and after centrifuging the suspension, the supernatant may be used as embryo culture medium. Conditioned medium may be stored at -70°C, if desired. Conditioned medium should be used at full strength for embryo culture (no dilution) (Eyestone (1989)).

## 2. Microinjection of hLF Transgene into Bovine Pronuclei

The DNA fragment containing the hLF expression unit is excised from the vector by digestion with the appropriate restriction enzyme(s) and separated by agarose gel electrophoresis. The fragment is purified by electroelution, phenol and chloroform extraction and ethanol precipitation. The DNA fragment is dissolved in and dialyzed in 10 mM Tris, 0.1 mM EDTA (pH 7.2) at a concentration of 1 to 2 µg/ml. Microinjection needles are filled with the dialyzed DNA solution.

Before in vitro fertilization, cumulus cells are removed from the egg by either vortexing at maximal speed for two minutes or pipetting up and down several times. Bovine pronuclei are injected in the same manner as murine pronuclei (Hogan et al., in: Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory (1986), incorporated herein by reference) with an additional centrifugation step in order to visualize

the pronuclei. The injection takes place 18-24 hours after fertilization. The time varies depending on the bull used as a source of semen. Nuclei become visible at different times in different batches of semen.

5           Bovine oocytes, matured and fertilized in vitro, are spun in a microfuge tube (Eppendorf) in 1 ml of tyrodes-hepes solution (Parrish (1987), incorporated herein by reference) at 14500xg for eight minutes (Wall et al., Biol. Reprod. 32:645-651 (1985), incorporated herein by reference). The embryos are  
10 transferred to a drop of tyrodes-hepes solution on a microscope slide covered with paraffin oil. Using a hydraulic system the oocytes are fixed to the egg holder in such a way that both the pronuclei are visible (using interference-contrast or phase contrast optics). If necessary, the oocytes are rolled to  
15 change their position on the egg holder to visualize the pronuclei. The injection needle is brought into the same sharp focus as one of the pronuclei. The needle is then advanced through the zona pellucida and cytoplasm into the pronucleus. A small volume, 1-3 pl, is injected (containing 20-100 DNA  
20 copies) into the pronucleus either by using a constant flow or a pulse flow (using a switch) of DNA solution out of the needle. Alternatively, two cell stage embryos are spun as described and the nuclei of both blastomers are injected as described. The injected embryos are then transferred to a drop  
25 of co-culture medium as described in Example 1 in order to develop to the morula or blastocyst stage.

### 3. Biopsies from developing embryos

30           In order to reproducibly and efficiently produce high quality embryos, oviduct cell conditioned medium was compared with oviduct epithelial cell coculture system as systems for culturing in vitro matured and fertilized oocytes up to the blastocyst stage. In the oviduct epithelial cell coculture system, embryos are cultured on a monolayer of bovine oviduct  
35 epithelial cells (BOEC). Using this system, 30% of the fertilized oocytes develop to the blastocyst stage.

The isolation of single or multiple blastomers from a preimplantation embryo (e.g., murine or bovine) requires

penetration of the zona pellucida (ZP). The murine ZP can easily be dissolved at a precise, limited area using a constant narrow flow of acidic Tyrode (pH 2.3) solution, although the ZP of bovine embryos cannot be dissolved by acidic Tyrode, even  
5 with a pH lower than 2.1.

Three other mechanical methods for ZP penetration have been tried to obtain biopsies from developing embryos:

- (1) splitting the complete embryo with very sharp blades;
- (2) making a hole in the ZP and pushing out single or multiple  
10 blastomers; and 3) making a slit in the ZP and aspirating blastomers with a beveled pipet.

(1) Splitting. This method is performed on later stage pre-implantation embryos, e.g., morulae and blastocysts. The advantages of this method are that it is very fast and  
15 simple and gives a relatively large number of cells for analysis. The disadvantages are that it is very crude method and a significant number of embryos will not survive. Also, the remaining part of the embryo has to be developed without ZP, which could be troublesome for continued culture, as the  
20 embryo tends to stick to the petri dish and/or BOEC monolayer), and in the transfer procedure.

The two other procedures are performed on 8 to 16 cell stage embryos. In these embryos the compaction process has not taken place and individual blastomers can still be  
25 distinguished. For the isolation of blastomers, the embryos do not need to be incubated in cytochalasin in order to disrupt cell-cell contracts. These procedures are performed as follows:

- (2) Making a hole in the ZP and pushing out  
30 blastomers. With a sharp glass needle a hole is made in the ZP and blastomer(s) can be pushed out using a blunt, polished glass rod. This method is also very fast and has some elegance in that the pushed out blastomer(s) will stick to the ZP on the outside and can undergo several cleavages. This facilitates  
35 the identification of blastomers and subsequent cleavage increases the number of cells that can be analyzed. The disadvantage of this method is that the pushing out of cells is

less precisely controlled: multiple cells or only parts of a single blastomer with or without a nucleus may be pushed out.

(3) Making a slit in the ZP and aspirating blastomers. In a preferred method, a slit is made in the ZP which can be opened with a beveled glass pipet with an opening large enough to subsequently aspirate blastomers. After withdrawing the aspiration pipet the ZP will reclose. This method is more laborious but can be more precisely controlled and causes the least disturbance of normal development. Little damage is caused to the embryo and to the blastomers that are removed.

These biopsy techniques are combined with the transgene detection methods described below. Biopsies obtained as described herein are also useful for multiplication of embryos proven to be transgenic.

#### 4. Early Detection of Transgenesis with hLF Transgene

Upon the microinjection of a construct, the oocyte is cultured, preferably at least to the 8-cell, even though analysis of embryos before that time is possible. A proper site of each embryo is cleaved and subjected to lysis (King et al., Molecular Reprod. and Devel. 1:57-62 (1988); proteolysis (Higuchi, "Amplifications (A Forum for PCR Users)" 2:1-3 (1989); both incorporated herein by reference) and Dpn I digestion. PCR is performed as described previously (Ninomiya et al., Molecular Reprod. and Devel. 1:242-248 (1989), incorporated herein by reference) with sets of two primers which flank a Dpn I site in the construct. One such set of primers are the forward primer ATG AAA CTT ATC CTC ACC TGT CTT GTG (in the  $\alpha$ S1 portion) and the reverse primer GGG TTT TCG AGG GTG CCC CCG AGG ATG GAT (in the hLF portion) of an hLF transgene disclosed in the commonly owned co-pending application U.S.S.N. 619,131, incorporated herein by reference.

#### 5. Early Detection of Transgenesis in Murine Embryos by PCR Analysis

72 hours old murine embryos resulting from a mating between a transgenic male and a nontransgenic female were

isolated. Two different males were used: one with a high transgene copy number (40-60) and the other with a low copy number (3). The number of cells in these embryos was around 50. About 50% of the embryos yielded a positive signal after  
5 PCR amplification.

To embryonic cells from blastomers or complete embryos in PBS (<5 $\mu$ l) are added 25 $\mu$ l H<sub>2</sub>O, and the cells are incubated 10 min at 95°C, then cooled slowly to room  
10 temperature. Depending on the number of cells use all or part of the sample is used in the following PCR reaction.

The following buffer is added to a total volume of 50 $\mu$ l in a 500 $\mu$ l microfuge (Eppendorf) tube: 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin, 0.1% Triton X-100, 50  $\mu$ M each of dATP, dGTP, dCTP and dTTP, 50 pmol  
15 forward primer, 50 pmol reverse primer, 0.8 U DpnI (Bethesda Research Laboratories), and 2 U Taq polymerase (Promega). The mixture is overlaid with a drop of light mineral oil to prevent evaporation. The mixture is incubated at 37°C for 20 min, followed by 93°C for 5 min. 50 cycles of PCR follow with each  
20 cycles consisting of: denaturation for 1 min at 93°C; annealing for 1 min at 55°C; and extension for 1.3 min at 72°C. After the 50 cycles are complete, a further 10 min incubation at 72°C follows. The reaction may be held at 4°C at this stage until further analysis is undertaken.

To analyze the PCR amplified products, about one-third to one-half of the PCR reaction volume is electrophoresed on an agarose or polyacrylamide gel. DNA bands are visualized by ethidium bromide staining, or, if additional sensitivity is required, by Southern blotting of the PCR products followed by  
25 probing with a labeled probe substantially complementary to the transgene.  
30

#### 6. Early Detection of Transgenesis in Bovine Embryos

By the following procedure 80% or more of the cells  
35 are synchronized in metaphase and suitable for analysis by in situ hybridization.

Chromosome Preparation from Preimplantation Bovine Embryos

(a) Synchronization. Cleavage bovine embryos are collected at 48 hours after in vitro fertilization and cultured for 6 hours in the presence of 0.1  $\mu$ g/ml colcemid (Sigma) by standard methods, e.g., 39°C in a gas phase of 50% CO<sub>2</sub> in air in drops of M199 medium (0.1% fetal calf serum, 0.01% antibiotic/antimycotic solution) under oil.

(b) Hypotonic treatment. The embryos are transferred to a watch glass containing 1.5 ml of precooled (4°C) 0.075 M KCl and kept at 4°C for 60 minutes.

(c) Fixation. Fixative (three/one, methanol/glacial acetic acid) is freshly prepared and kept at -20°C until needed. Three ml of precooled fixative is placed in a precooled thick watch glass and the embryos are added. It is important that the watch glass is thick enough to maintain the temperature of the fixative reasonably constant during ensuing operation under the microscope. If the temperature rises quickly, two problems arise: (1) most of the embryos will burst, (2) the embryos do not remain sunken at the bottom of the watch glass but float around in the fixative, making it impossible to find and collect them under the microscope. The fixation time is approximately 30 minutes at -20°C.

(d) Spreading. Each embryo is removed from the watch glass and placed with not more than 1  $\mu$ l fixative on a grease-free slide (preringed on the back with a diamond pencil) under a stereo-microscope. Before the fixative completely evaporates, not more than one  $\mu$ l of softening solution (equal parts of methanol and 75% acetic acid, precooled to 4°C) is added at room temperature and carefully observed to monitor the degree of spreading. Application of the softening solution is continued until the spread is satisfactory.

Pre-Hybridization Treatment of Slides

Before the initiation of hybridization procedure an individual metaphase spread on each slide is located by phase-contrast microscopy and recorded for speedy detection of the metaphase spread after the in situ hybridization procedure.

(a) RNAse treatment. Each slide is incubated with 100  $\mu$ l RNAse (0.1 mg/ml) under a coverslip for 60 minutes at 37°C in a slide-jar. At the end of incubation, the coverslip is removed by the addition of 100 ml of 2xSSC (Sambrook et al., 1989) and gentle shaking of the slide-jar for 5 minutes on the top of an automatic shaker. The slides are further washed three times with 2xSSC at room temperature. Throughout the entire procedure, all the washings require 5 minutes incubation at room temperature with constant gentle shaking unless specified otherwise. Slides are dehydrated by sequential washing with 70%, 90%, and 100% ethanol.

(b) Pepsin treatment. The slides are incubated in 100 ml of pepsin (0.1 mg/ml 0.01 M HCl) in a slide-jar for 10 minutes at 37°C, followed by two washings with PBS.

(c) MgCl<sub>2</sub> treatment and fixation. The slides are incubated for 5 minutes with 50 mM MgCl<sub>2</sub>/PBS in a slide-jar at room temperature, followed by incubation for 10 min with 1.0% formaldehyde/50 mM MgCl<sub>2</sub>/PBS at room temperature. The slides are washed once with PBS and dehydrated by washing with 70%, 90%, and 100% ethanol.

#### Denaturation, Probe Preparation, and Hybridization.

(a) Denaturation. Chromosomes are denatured by incubation of the slides for 3 minutes at 80°C after application of 100  $\mu$ l of denaturation solution (a mixture of 70  $\mu$ l of 100 formamide, 10  $\mu$ l of 20xSSC and 10  $\mu$ l of 0.5 M phosphate, pH 7.0 and 10  $\mu$ l of H<sub>2</sub>O) per each slide and covered with coverslips. After denaturation, 100 ml of chilled (-20°C) 70% ethanol was poured into the slide-jar and the slides are dehydrated and air-dried at room temperature.

(b) Probe Preparation. One  $\mu$ g of DNA for the probe is digested with DNase (0.001 ng/ $\mu$ l) overnight at 37°C, then nick-translated in the presence of 40  $\mu$ M biotin-11-dUTP (Sigma) for 1 hour at 14°C. The reaction is stopped by addition of 0.5 M EDTA. The labelled probe is purified by running on a G-50 sephadex column. The degree of labelling of the probe is monitored by using Blugene (BRL), a nonradioactive nucleic acid detection system. Probe (20 ng per slide) and sheared bovine

genomic DNA (10 µg per slide) is dissolved in a desired volume of hybridization solution (a mixture of 2.5 µl 100% formamide, 0.5 µl 20xSSC, 0.5 µl 0.5 M phosphate (pH 7.0), 1.5 µl H<sub>2</sub>O and 5 µl 20% dextran sulfate. The probe solution is then denatured by heating at 75°C for 5 minutes followed by quick chilling to 4°C. The probe is further incubated for at least 2 hours at 37°C to block out repetitive sequences before application to slides (10 µl per slide).

(c) Hybridization. Coverslips are boiled for 20 minutes in 0.2 M HCl and washed four times each with tap water, deionized water, distilled water, and 100% ethanol. The coverslips are dried and baked for at least 2 hours at 180°C. The slides are covered with coverslips after application of 10 µl of probe, sealed with glue (Simson), and incubated for approximately 16 hours at 37°C.

Post-Hybridization Washing.

The coverslips loosened by 100 ml of washing solution A (50% formamide/2xSSC, adjusted to pH 7.0 with HCl and prewarmed to 45°C). After coverslips are removed, the slides are washed three times with solution A.

The slides are further washed three more times with washing solution B (0.1xSSC, 60°C). As described previously, each washing takes 5 minutes with shaking of the slide-jar. It is important that the temperature is maintained either at 45°C or 60°C throughout the washing procedure. One additional washing is carried out with washing solution C (4xSSC/0.05% Tween 20) at room temperature.

30 Immunocytochemical amplification

(a) Blocking. Each slide is incubated with 100 µl of blocking solution (4xSSC/5% non-fat dry milk) with a coverslip for 10 minutes at room temperature. The coverslip is removed by shaking the slide-jar with 100 ml of washing solution C.

(b) Immunocytochemical Reactions. One hundred µl of fluorescein avidin-D FITC (Vector Lab) (diluted to 5 µg/ml with blocking solution, followed by spinning in a microfuge for 1.5

minutes to remove dust particles) is applied to each slide, covered with a coverslip, and incubated for 20 minutes at room temperature. The coverslip is removed by shaking the slide-jar with 100 ml of washing solution C. Slides are washed two  
5 additional times with washing solution C.

This incubation and washing procedures are repeated with 100  $\mu$ l of biotinylated goat-anti-avidin D (Vector Lab) (diluted to 5  $\mu$ g/ml with blocking solution and centrifuged) per  
slide.

10 The third immunocytochemical reaction is carried out with fluorescein avidin-D FITC solution as described above.

The slides are dehydrated by washing with 100 ml of 70%, 90%, and 100% ethanol.

#### 15 Embedding

Embedding solution is made by dissolving 2 gm of 1,4-Diazabicyclo-(2,2,2)-octane (DABCO) in 90 ml of glycerol for 15-30 minutes at 60°C and 10 ml of 1.0 M Tris-HCl (pH 7.5) is added and the pH is adjusted to 8.0 with a few drops of 5 M  
20 HCl. The solution is cooled to room temperature before the addition of 100  $\mu$ l of Thimerosal solution (20%) and 100  $\mu$ l of propidium iodide solution (1 mg/ml). 35  $\mu$ l embedding solution is applied to each slide and covered with a coverslip before fluorescent-microscopical (Olympus) examination.

25

#### Early Detection of Transgenesis in Murine Embryos by In Situ Analysis

Two to three day old embryos from a mating between a hLF transgenic male and a non-transgenic female were  
30 synchronized with colcemid for 4 hours. About 15% of the cells (usually  $\pm 3$  out of 20) are arrested in metaphase. In situ hybridization on the metaphase spreads with a biotinylated hLF cDNA construct (by the procedure described above) showed that 40-50% of the embryos are hLF positive as manifested by  
35 visualization of two clear fluorescent spots at identical locations in the sister chromatids. The positive identification was possible in embryos derived from mating of a transgenic male carrying only a single copy hLF cDNA transgene.

Next, embryos were analyzed which were derived from non-transgenic parents but injected in the pronuclear stage with hLF cDNA constructs. As described above, double fluorescent spots could be detected in a number of embryos, but  
5 in the presence of a highly fluorescent background. We believe the background originated from injected DNA which is neither incorporated in nor cleared from the nuclei. The combination of the background fluorescence and the low number of metaphase spreads per embryo does not allow unambiguous identification of  
10 the transgenic embryos.

In the third set of experiments the number of metaphase spreads per embryo was increased. Control embryos (3 days old) were synchronized in the S-phase using varying concentrations of colchicine before being release to and  
15 subsequent blocked in mitosis. Incubation for 16 hours in a low concentration of colchicine (<10 ng/ml) resulted in the highest mitotic index, i.e., 75% metaphase spreads per embryo (15 out of 20 cells).

While the invention has been described in connection  
20 with certain embodiments thereof, it should be recognized that various modifications as may be apparent to one of skill in the art to which the invention pertains also fall within the scope of the invention as defined by the appended claims. The scope of the invention should, therefore, be determined not with  
25 reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

## WHAT IS CLAIMED IS:

1. A method for the detection of an integrated transgene in the nuclear genome of an embryo of an animal, said method  
5 comprising the steps of:
  - a) performing in situ hybridization on at least one metaphase stage cell from said embryo with a nucleic acid probe substantially complementary to said transgene; and
  - b) detecting a hybridization signal on both sister  
10 chromatids of a chromosome of said cell.
2. A method of claim 1 further comprising the step of removing said metaphase stage cell from said embryo prior to said in situ hybridization step, thereby producing a viable  
15 biopsied embryo.
3. A method of claim 1 wherein said embryo is an 8 to 16 cell embryo.
- 20 4. A method of claim 1 wherein said probe is biotinylated.
5. A method of claim 1 wherein said probe is at least about 2 kilobases.
- 25 6. A method of claim 1 wherein said animal is a bovine species.
7. A method of claim 1 further comprising the step of determining the sex of said embryo.  
30
8. A method for the detection of an integrated transgene in the nuclear genome of an 8 to 16 cell embryo of an animal, said method comprising the steps of:
  - a) removing at least one metaphase stage cell from  
35 said embryo, thereby producing a viable biopsied embryo;
  - b) performing in situ hybridization on said at least one metaphase stage cell from said embryo with a nucleic acid probe substantially complementary to said transgene; and

c) detecting a hybridization signal on both sister chromatids of a chromosome of said cell.

9. A method of claim 8 further comprising the step of  
5 determining the sex of said embryo.

10. A viable biopsied embryo produced according to any of claims 1-9.

10 11. A method for the detection of an integrated transgene in the nuclear genome of an embryo of an animal, said method comprising the steps of:

a) cloning said embryo, thereby producing a multiplicity of cloned embryos;

15 b) performing in situ hybridization on at least one metaphase stage cell from said embryo with a nucleic acid probe substantially complementary to said transgene; and

c) detecting a hybridization signal on both sister chromatids of a chromosome of said cell.

20

12. A method of claim 11 further comprising the step of removing at least one cell from said embryo, thereby producing a viable biopsied embryo.

25 13. A method of claim 11 wherein said embryo is an 8 to 16 cell embryo.

14. A method of claim 11 wherein said probe is biotinylated.

30 15. A method of claim 11 wherein said step of cloning is by nuclear transfer.

16. A method of claim 11 wherein said probe is at least about two kilobases.

35

17. A method of claim 11 wherein said animal is a bovine species.

18. A method for the detection of an integrated transgene in the nuclear genome of an 8 to 16 cell embryo of an animal, said method comprising the steps of:

- a) cloning said embryo by nuclear transfer, thereby  
5 producing a multiplicity of cloned embryos;
- b) removing at least one metaphase stage cell from at least one said cloned embryos, thereby producing a viable biopsied embryo;
- c) performing in situ hybridization on said at least  
10 one metaphase stage cell with a nucleic acid probe substantially complementary to said transgene; and
- d) detecting a hybridization signal on both sister chromatids of a chromosome of said cell.

15 19. A method for the detection of an integrated transgene in the nuclear genome of an 8 to 16 cell bovine embryo, said method comprising the steps of:

- a) cloning said embryo by nuclear transfer, thereby  
producing a multiplicity of cloned embryos;
- 20 b) removing at least one metaphase stage cell from at least one of said cloned embryos, thereby producing a viable biopsied embryo;
- c) performing in situ hybridization on said at least  
one metaphase stage cell with a biotinylated nucleic acid probe  
25 substantially complementary to said transgene; and
- d) detecting a hybridization signal on both sister chromatids of a chromosome of said cell.

20. A method of claim 11, 18, or 19, further comprising the  
30 step of determining the sex of the embryo.

21. A viable biopsied embryo produced according to any of claims 11, 18, 19, or 20.

35 22. A method for detecting integration of a transgene into the nuclear DNA of an embryo of an animal and replication of said transgene as part of said nuclear DNA, wherein said integration and replication provides for a methylated restriction site of

said transgene becoming an unmethylated restriction site, said method comprising the steps of:

- a) treating said nuclear DNA from at least one cell from said embryo with a restriction enzyme capable of cleaving said methylated restriction site and incapable of cleaving said unmethylated restriction site, thereby producing restriction fragments of said nuclear DNA;
- b) amplifying said restriction fragments by PCR with PCR primers substantially complementary to sequences on different strands of said transgene and flanking said unmethylated restriction site, thereby forming an amplified transgene; and
- c) detecting said amplified transgene.

23. A method of claim 22 wherein said PCR primers comprise sequences other than ATGAAACTTATCCTCACCTGTCTTGTG or GGGTTTTTCGAGGGTGCCCCGAGGATGGAT.

24. A method of claim 22 further comprising the step of removing at least one cell from said embryo, thereby producing a viable biopsied embryo.

25. A method of claim 22 wherein said restriction enzyme is DpnI and said methylated restriction site is methylated at N6 of an adenine of said methylated restriction site.

26. A method of claim 22 wherein said embryo is an 8 to 16 cell embryo.

27. A method of claim 22 wherein said animal is a bovine species.

28. A method of claim 22 wherein said PCR primers comprise at least about 16 nucleotides.

29. A method of claim 22 further comprising the steps of:

performing in situ hybridization on at least one metaphase stage cell from said embryo with a nucleic acid probe substantially complementary to said transgene; and

5 detecting a hybridization signal on both sister chromatids of a chromosome of said cell.

30. A method of claims 22-29, further comprising the step of determining the sex of said embryo.

10 31. A method for detecting integration of a transgene into the nuclear DNA of an 8 to 16 cell embryo of an animal and replication of said transgene as part of said nuclear DNA, wherein said integration and replication provides for a methylated restriction site of said transgene becoming an  
15 unmethylated restriction site, said method comprising the steps of:

a) removing at least one cell from said embryo, thereby producing a viable biopsied embryo;

20 b) treating said nuclear DNA from said removed cell with a restriction enzyme capable of cleaving said methylated restriction site and incapable of cleaving said unmethylated restriction site, thereby producing restriction fragments of said nuclear DNA;

25 c) amplifying said restriction fragments by PCR with PCR primers substantially complementary to sequences on different strands of said transgene and flanking said unmethylated restriction site, thereby forming an amplified transgene; and

30 d) detecting said amplified transgene.

32. A viable biopsied embryo produced according to any of claims 22-31.

35 33. A method for detecting a transgene in an embryo of an animal, said method comprising the steps of:

a) cloning said embryo, thereby producing a multiplicity of cloned embryos;

b) amplifying DNA from at least one cell of at least one of said cloned embryos by PCR, thereby forming an amplified transgene; and

c) detecting said amplified transgene.

5

34. A method of claim 33 wherein:

said transgene is integrated into the nuclear genome of said animal and replicated as part of said genome, and said integration and replication provides for a methylated

10 restriction site of said transgene becoming an unmethylated restriction site;

said step of performing PCR employs PCR primers substantially complementary to sequences on different strands of said transgene and flanking said unmethylated restriction  
15 site; and

said method further comprises the step of treating said nuclear DNA from at least one cell of at least one of said cloned embryos with a restriction enzyme capable of cleaving said methylated restriction site and incapable of cleaving said  
20 unmethylated restriction site.

35. A method of claim 33 wherein said restriction enzyme is Dpn I and said methylated restriction site is methylated at N6 of an adenine of said methylated restriction site.

25

36. A method of claim 33 further comprising the step of removing at least one cell from at least one of said cloned embryos, thereby producing a viable biopsied embryo.

30 37. A method of claim 33 wherein said step of amplifying DNA further comprises the steps of:

cleaving said DNA with a restriction enzyme incapable of cutting within said transgene, thereby producing restriction fragments of said DNA;

35

adding ligase to said restriction fragments under conditions which result in self ligation of said restriction fragments, thereby producing circularized restriction fragments;

amplifying said circularized restriction fragments by inverse PCR, thereby producing an amplified transgene; and detecting said amplified transgene.

5 38. A method of claim 33 wherein said step of cloning is by nuclear transfer.

39. A method of claim 33 wherein said embryo is an 8 to 16 cell embryo.

10

40. A method of claim 33 wherein said animal is a bovine species.

41. A method of claim 40, wherein said animal is a cow.

15

42. A method of claim 33 wherein said PCR primers comprise about 16 nucleotides.

43. A method according to claims 33-41, further comprising the  
20 step of determining the sex of the embryo.

44. A method for detecting integration of a transgene into the nuclear DNA of an 8 to 16 cell embryo of an animal and replication of said transgene as part of said nuclear DNA,  
25 wherein said integration and replication provides for a methylated restriction site of said transgene becoming an unmethylated restriction site, said method comprising the steps of:

- 30 a) cloning said embryo by nuclear transfer, thereby producing a multiplicity of cloned embryos;
- b) removing at least one cell from said cloned embryos, thereby producing at least one viable biopsied embryo;
- c) treating said nuclear DNA from said removed cell  
35 with a restriction enzyme capable of cleaving said methylated restriction site and incapable of cleaving said unmethylated restriction site, thereby producing restriction fragments of said nuclear DNA;

- d) amplifying said restriction fragments by PCR with PCR primers substantially complementary to sequences on different strands of said transgene and flanking said unmethylated restriction site, thereby forming an amplified transgene; and
- e) detecting said amplified transgene.

45. A method for detecting integration of a transgene into the nuclear DNA of an 8 to 16 cell embryo of a bovine species and replication of said transgene as part of said nuclear DNA, wherein said integration and replication provides for a methylated Dpn I restriction site of said transgene becoming an unmethylated Dpn I restriction site, said method comprising the steps of:

- a) cloning said embryo by nuclear transfer, thereby producing a multiplicity of cloned embryos;
- b) removing at least one cell from said cloned embryos, thereby producing at least one viable biopsied embryo;
- c) treating said nuclear DNA from said removed cell with Dpn I, thereby producing Dpn I restriction fragments of said nuclear DNA;
- d) amplifying said Dpn I restriction fragments by PCR with PCR primers substantially complementary to sequences on different strands of said transgene and flanking said Dpn I restriction site, thereby forming an amplified transgene; and
- e) detecting said amplified transgene.

46. A viable biopsied embryo produced according to any of claims 33-44.

30

47. A method for detecting integration of a transgene into the nuclear DNA of an embryo of an animal and replication of said transgene as part of said nuclear DNA, wherein said integration and replication provides for a methylated restriction site of said transgene becoming an unmethylated restriction site, said method comprising the steps of:

- a) treating said nuclear DNA from at least one cell from said embryo with a restriction enzyme capable of cleaving

said methylated restriction site and incapable of cleaving said unmethylated restriction site, thereby producing restriction fragments of said nuclear DNA;

5 b) amplifying said restriction fragments by PCR with PCR primers substantially complementary to sequences on different strands of said transgene and flanking said unmethylated restriction site, thereby forming an amplified transgene;

10 c) detecting said amplified transgene;  
d) performing in situ hybridization on at least one metaphase stage cell from said embryo with a nucleic acid probe substantially complementary to said transgene; and  
e) detecting a hybridization signal on both sister chromatids of a chromosome of said cell.

15

48. A method for detecting a transgene in an embryo of an animal, said method comprising the steps of:

a) cloning said embryo, thereby producing a multiplicity of cloned embryos;  
20 b) amplifying DNA from at least one cell of said cloned embryos by PCR, thereby forming an amplified transgene;  
c) detecting said amplified transgene;  
d) performing in situ hybridization at least one metaphase stage cells from said cloned embryos with a nucleic  
25 acid probe substantially complementary to said transgene; and  
e) detecting a hybridization signal on both sister chromatids of a chromosome of said cell.

49. A method according to claims 44-48, further comprising the  
30 step of determining the sex of the embryo.

50. A viable embryo produced according to any of claims 44-49.

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US91/04149**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12Q 1/68; C12P 19/34; C12N 15/00; C07H 15/12; A01H 5/00 U.S.CL: 435/6,91, 172.3; 800/2; 536/27																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black; text-align: left;">Classification System</th> <th style="border: 1px solid black; text-align: left;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">U.S.</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">435/6,91,172.3; 800/2;536/27</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div> <p style="text-align: center; padding: 5px;">APS, CAS Data Bases</p>			Classification System	Classification Symbols	U.S.	435/6,91,172.3; 800/2;536/27											
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 5%; border: 1px solid black; text-align: left;">Category <sup>*</sup></th> <th style="width: 75%; border: 1px solid black; text-align: left;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; border: 1px solid black; text-align: left;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">Y</td> <td style="border: 1px solid black; vertical-align: top;">Cell, volume 49, issued 1987 Crenshaw et al., "Neuron in Transgenic Mice Expressing a Metallothionein-Calcitonin Fusion Gene" pages 389-398, see figures 5,6 and 7.</td> <td style="border: 1px solid black; vertical-align: top;">1-50</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">Y</td> <td style="border: 1px solid black; vertical-align: top;">Nature, volume 335, issued 1988, Li et al., "Amplification and analysis of DNA sequence in single human sperm and diploid cells" page 414-417, see abstract.</td> <td style="border: 1px solid black; vertical-align: top;">1-50</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">Y</td> <td style="border: 1px solid black; vertical-align: top;">Proc. Natl. Accad. Scie, vol. 86, issued 1989, Cui et al., "Single sperm typing: Determination of generic distance between the G gamma-globin and parathyroid hormone loci by using the polymerase chain reaction and allele specific oligomer", pages 9389-9393, see abstract.</td> <td style="border: 1px solid black; vertical-align: top;">1-50</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">Y</td> <td style="border: 1px solid black; vertical-align: top;">Chemical Abstracts Volume 113, issued 1990, King et al., "Analyzing embryos by the polymerase chain reaction" abstract No. 185758 W, UCLA Symp. Mol Biol (1990) 116, 33-35 see abstract.</td> <td style="border: 1px solid black; vertical-align: top;">1-50</td> </tr> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	Y	Cell, volume 49, issued 1987 Crenshaw et al., "Neuron in Transgenic Mice Expressing a Metallothionein-Calcitonin Fusion Gene" pages 389-398, see figures 5,6 and 7.	1-50	Y	Nature, volume 335, issued 1988, Li et al., "Amplification and analysis of DNA sequence in single human sperm and diploid cells" page 414-417, see abstract.	1-50	Y	Proc. Natl. Accad. Scie, vol. 86, issued 1989, Cui et al., "Single sperm typing: Determination of generic distance between the G gamma-globin and parathyroid hormone loci by using the polymerase chain reaction and allele specific oligomer", pages 9389-9393, see abstract.	1-50	Y	Chemical Abstracts Volume 113, issued 1990, King et al., "Analyzing embryos by the polymerase chain reaction" abstract No. 185758 W, UCLA Symp. Mol Biol (1990) 116, 33-35 see abstract.	1-50
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																	
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; vertical-align: top; padding: 5px;">           Date of the Actual Completion of the International Search   <b>9/11/91</b> </td> <td style="width: 50%; border: 1px solid black; vertical-align: top; padding: 5px;">           Date of Mailing of this International Search Report   <b>25 SEP 1991</b> </td> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">           International Searching Authority   <b>ISA/US</b> </td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">           Signature of Authorized Officer   <b>Scott A. Chambers</b> </td> </tr> </table>			Date of the Actual Completion of the International Search  <b>9/11/91</b>	Date of Mailing of this International Search Report  <b>25 SEP 1991</b>	International Searching Authority  <b>ISA/US</b>	Signature of Authorized Officer  <b>Scott A. Chambers</b>											
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## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Chemical Abstracts, volume 110, issued 1989, King et al., "Identification of specific gene sequence in preimplantation embryos by genomic amplification: detection of a transgene (1988) 1(1) 57-62, See abstract.	1-50
Y	Chemical Abstracts, volume 111 issued 1989, Ninomiya et al., "Selection of mouse preimplantation embryos carrying exogenous DNA by polymerase chain reaction" abstract No. 210082q; Mol. Reprod. Dev (1989) 1(4) 242-8 see abstract.	1-50

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter<sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>13</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US92/05097 <b>(22) International Filing Date:</b> 12 June 1992 (12.06.92) <b>(30) Priority data:</b> PCT/US91/04149 12 June 1991 (12.06.91) WO <b>(34) Countries for which the regional or international application was filed:</b> AT et al. <b>(71) Applicant (for all designated States except US):</b> GEN-PHARM INTERNATIONAL, INC. [US/US]; 2375 Garcia Avenue, Mountain View, CA 94043 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KRIMPENFORT, Paul [NL/NL]; Crayenestersingel 5, NL-2101 AN Heemstede (NL). LEE, Sang, He [US/NL]; Sotaweg 24, NL-2371 GD Roelofarendsveen (NL). STRIJKER, Rein [NL/NL]; Spaaragarenstraat 2, NL-2341 JW Oegstgeest (NL).		<b>(74) Agents:</b> SMITH, William, M et al.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US). <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i>
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<b>(57) Abstract</b>		
<p>The present invention provides methods, based on <i>in situ</i> hybridization and the polymerase chain reaction, for the early detection of integrated transgenes in the nuclear genome.</p>		